

Methods for the Study of Programmed Cell Death in the Nematode *Caenorhabditis elegans*

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Abstract

The nematode *Caenorhabditis elegans* has been shown to be an excellent model organism with which to study the mechanisms of programmed cell death because of its powerful genetics and the ability to study cell death with single cell resolution. In this article, we describe methods that are commonly used to examine various aspects of programmed cell death in *C. elegans*. These methods, in combination with genetic analyses, have helped identify and characterize many components of the *C. elegans* cell death pathway, illuminating the mechanisms by which these components affect programmed cell death.

Introduction

Programmed cell death is a widespread phenomenon in the animal kingdom and a prominent feature in the development of the nematode *C. elegans*.¹ During the development of an adult hermaphrodite, 131 out of 1090 somatic cells generated undergo programmed cell death.^{2, 3, 4} The ability to observe and follow cell division or cell death of individual cells in transparent living animals using Nomarski Differential Interface Contrast (DIC) optics facilitated the determination of the complete *C. elegans* cell lineage^{2, 3, 4} and the determination of the exact time and position of the 131 cells that die, which are invariant from animal to animal.^{2, 4} The advantage of studying cell death at the single cell level in combination with the powerful genetics available in *C. elegans* has led to the identification of many mutations that affect different aspects of programmed cell death and has helped define a genetic pathway that regulates and executes programmed cell death in *C. elegans*.¹ Included in this pathway are genes that specify which cells should die (e.g., *ces-1* and *ces-2*),⁵ genes that execute the cell-death program (e.g., *ced-3*, *ced-4*, and *ced-9*),^{6, 7} genes that are involved in cell-corpse engulfment (e.g.,

ced-1, *ced-2*, *ced-5*, *ced-6*, *ced-7*, and *ced-10*),^{8,9} and genes that clean up cellular debris (e.g., *nuc-1*).¹⁰

A second advantage of studying cell death in *C. elegans* is the availability of a detailed genetic map, the physical map of cosmid and YAC clones that cover the entire genome,^{11,12} and the almost completely sequenced genome.¹² Such tools greatly facilitate the cloning and the characterization of cell-death genes of interest. Furthermore, the emergence of the powerful double-stranded RNA (dsRNA) interference technique to produce potential loss-of-function phenotypes in specific genes¹³ and the ease of isolation of deletion mutations from libraries of mutagenized worms using PCR based screening¹⁴ makes reverse genetics a practical approach to determine the functions of *C. elegans* homologs of mammalian cell-death proteins. In this article, we review and describe the methods that one can use to study various features of programmed cell death in *C. elegans*. General techniques for culturing and handling of *C. elegans* have been described in detail in other references.^{15, 16, 17}

Description of Methods

Assays for Scoring Cell Survival Using Nomarski Optics

The transparent nature of *C. elegans* cuticle allows cells to be observed using Nomarski optics. Since the *C. elegans* cell lineage has been defined and is invariant from animal to animal, it is possible to identify and characterize mutations that block programmed cell death by scoring extra surviving cells.^{5,6,7} The anterior pharynx, a well defined structure,¹⁸ is especially suited for counting extra surviving cells (Figure 1).⁹ Nuclei of the cells in the anterior pharynx are easily recognized as rippled indents in the smooth wall of the pharynx (Figure 1B and 1C). Sixteen cells in the anterior pharynx of wild-type animals undergo programmed cell death during mid-

embryogenesis; their corpses are rapidly engulfed and disappear.⁴ In cell-death defective mutants, such as *ced-3* and *ced-4* mutants, some of the sixteen cells survive and the resulting extra nuclei are readily visible and can be counted under Nomarski optics (Figures 1B and 1C). The number of extra nuclei in the anterior pharynx reflects the severity of the cell-death defect in mutant animals. For example, in a strong *ced-3(n2433)* mutant, an average of 12.4 extra nuclei (extra cells) are seen in the anterior pharynx, while in a weak *ced-3(n2427)* mutant, an average of only 1.2 extra cells are seen.¹⁹

To score pharyngeal nuclei, the third (L3) or the fourth (L4) stage larvae are anesthetized by mounting on an agar pad with a drop of 10 mM sodium azide (NaN₃) solution and the number of extra nuclei in the anterior pharynx are counted using Nomarski microscopy. Figure 1A schematically shows the positions of the sixteen cells (nuclei) that undergo programmed cell death in the anterior pharynx of wild-type animals.^{9, 18}

Two serotonergic NSM neurons are located at the posterior part of the anterior pharynx. Their sister cells normally undergo programmed cell death (Figure 1).^{4, 5} Gain-of-function mutations in the *ces-1* gene or a loss-of-function mutation in the *ces-2* gene prevent two NSM sister cells from undergoing programmed cell death.⁵ In these mutants or other cell-death defective mutants (e.g., *ced-3* and *ced-4* mutants), an extra nucleus of the NSM sister cell can be observed just posterior and dorsal to each of two NSM neurons (Figure 1C). This survival vs. death assay for NSM sister cells presents an example of how programmed cell death can be studied in a single cell in *C. elegans*.

Cell Corpse Assay For Defects in Cell Death

Most somatic cell deaths (113/131) in *C. elegans* hermaphrodites occur during embryogenesis, mainly between 250 to 450 minutes after fertilization.^{2,4} The remainder of the cell deaths occur during the first (L1) and second (L2) larval stages outside the head region of the animals.² Like somatic cells, some cells in the hermaphrodite germline also undergo programmed cell death. The germline is syncytial; however, during programmed cell death, germline nuclei that are destined to die become cellularized.²⁰ Cell deaths in the germline appear to occur in the region where nuclei are arrested in the pachytene stage of meiosis, near the turn of the gonadal arm (Figure 2B), but not in the mitotic region.²⁰ Occasionally, mature oocytes have been observed to undergo programmed cell death.

When cells in *C. elegans* undergo programmed cell death, they adopt refractile and raised-button-like morphology that persists for 10 to 30 minutes as observed under Nomarski optics (Figure 2). Since the same cells always die at characteristic times in every wild-type animal, mutations that affect cell death can be easily identified and characterized based upon altered patterns or morphology of cell-corpses.

Many mutations have been found to generate abnormal cell-corpse patterns. For example, mutations in the *ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, or *ced-10* genes result in persisting cell corpses, suggesting that these genes are involved in either the recognition or the phagocytosis of cell corpses during the engulfment process.^{8,9} The persistent cell corpse phenotype is easily distinguished in the head region of early larvae as no larval cell deaths normally occur in this region; corpses observed are those that have been generated during embryogenesis and persist through early larval stages (Figure 3). By contrast, in animals that are defective in cell-death execution such as *ced-3* and *ced-4* mutants, the number of cell corpses at all developmental stages is reduced since cells that normally die now survive. Therefore, the

cell-corpse assay is useful for assessing defects in the killing or the engulfment process of programmed cell death.

Cell corpses in a specific region and at a particular developmental stage can be counted to quantitate the severity of cell-death defects. For example, the expressivity and the penetrance of the engulfment-defective mutants has been quantitated by scoring the number of cell corpses in the pharynx of L1 larvae.⁹

Since cell corpses in *C. elegans* all have a characteristic raised-button-like morphology (Figure 2), mutations that affect the morphology of cell corpses could be identified. Furthermore, mutations that affect the timing or kinetics of cell death may change the time course of corpse appearance.²⁰

Methods for Viewing C. elegans Using Nomarski DIC Optics and Recovery of Animals from Slides

The above methods for scoring extra surviving cells and cell corpses involve viewing *C. elegans* using Nomarski microscopy. Animals are placed on top of an agar pad containing a drop of 10 mM NaN_3 solution on a microscope slide. Sodium azide anesthetizes the animals so that individual cells or cell corpses can be observed with high magnification objectives without being disturbed by worm movement. This procedure also allows individual animals or embryos to be rescued alive off the agar pad. Using this method, populations of nematodes can be screened for subtle cell-death defective phenotypes that normally would not be detected under the dissecting microscope.

To make an agar pad, melt 5% agar in M9 solution (40 mM Na_2HPO_4 , 22 mM KH_2PO_4 , 8.5 mM NaCl , 18.7 mM NH_4Cl) and keep it at 65°C in a heating block. A tape of approximately

0.1 mm in thickness is applied to two slides, which are used as spacer slides. The specimen slide is placed in parallel between the two-spacer slides flat on a desk and a small drop of melted agar is then spotted in the middle of the specimen slide. A fourth slide is immediately pressed down perpendicular to all three slides, onto the tape of the spacer slides. The drop of agar is squashed flat, and the specimen slide is then carefully slid out from underneath of the fourth slide (Figure 4). 1-2 μ l of 10 mM NaN_3 in M9 solution is then spotted on the agar pad, and embryos or worms are placed in the sodium azide solution. Sodium azide anesthetizes the worms without killing them, so that they can be rescued later if desired. To view embryos, M9 buffer is usually used instead of the sodium azide solution as eggs are immobile. A coverslip is gently laid on the top of the agar pad. To view living cells or cell corpses, Nomarski DIC optics is required, usually at 1000x magnification.

To rescue live worms or embryos from the agar pad, the positions of the worms or embryos of interest on the microscope slide are recorded using the positions of other worms or embryos as references and the slide is taken to the dissecting scope. The coverslip is carefully removed until the desired worm or embryo is exposed. A mouth pipette filled with M9 solution is used to transfer the worm or embryo to a clean plate seeded with bacteria. A small amount of M9 is drawn into the fine end of the mouth pipette via capillary action. Then the mouth piece that comes with the capillary tubes is used to expel a small drop of M9 directly onto the worm. The M9 solution is then drawn back into the mouth pipette along with the worm or embryo and expelled again onto a clean plate. To make a mouth pipette, a 50 μ l capillary tube is pulled into a needle shape via heating the middle of the tube and then quickly pulling two ends of the capillary tube apart from each other. The capillary tube is then broken at the middle and the fine end of the tube is used to transfer worms.

Assays for Defects in DNA Degradation

One conserved feature of programmed cell death is the degradation of genomic DNA of dying cells.¹ The degradation of DNA in dying cells of *C. elegans* can be monitored by staining animals *in situ* with a DNA labeling dye, such as DAPI (diamidinophenolindole) or Feulgen.^{8, 10}

Cell deaths in *C. elegans* ventral cord occur only during the first (L1) and the second (L2) larval stages of development.² When wild-type larvae at L3 or L4 developmental stages are stained with Feulgen, living cells but not the cells that have died earlier in the ventral cord show Feulgen staining.⁸ By contrast, in mutants that are defective in cell-corpse engulfment, some cell corpses in the ventral cord persist through late larval stages and show extra Feulgen staining.⁸ This observation indicates that engulfment of cell corpses is a prerequisite for complete degradation of genomic DNA of dead cells. Like engulfment-defective mutants, late larvae of *nuc-1* mutant animals also exhibit extra Feulgen staining at positions where cell deaths have occurred. In *nuc-1* mutants, both cell killing and engulfment processes proceed normally but the pycnotic DNA of dead cells is not completely degraded and persists as a compact mass of DAPI- or Feulgen-reactive material^{8, 10}. The *nuc-1* gene is also required for the degradation of genomic DNA from germline cell corpses: large DAPI-positive DNA masses accumulate in the sheath cells (the engulfing cells for germline cell corpses) of *nuc-1* mutant animals but not in those of wild-type animals.²⁰ In addition, *nuc-1* mutant animals show persistent DAPI staining in the intestinal lumen since the genomic DNA from the bacteria on which worms are fed is not completely degraded in the intestine of the *nuc-1* mutants.^{8, 10}

To perform a DAPI DNA staining assay, animals are harvested from plates using M9 buffer and washed twice with the same buffer to remove bacteria. They are then transferred to a

microcentrifuge tube containing 1 ml fixative solution (60% ethanol, 30% acetic acid, and 10% chloroform) and fixed for at least 1.5 hours with mild agitation. The fixed worms are then washed twice with M9 buffer and stained in DAPI staining solution (1.0 µg/ml DAPI in M9 buffer) for at least 30 minutes. The stained animals are mounted on an agar pad for observation using a fluorescent microscope with a DAPI filter. The Feulgen staining assay is more laborious and thus less frequently used. The procedures for the Feulgen staining have been described in detail by Sulston and Horvitz (1977).²

The DNA staining assays are useful for identifying and characterizing phenotypes involving abnormal DNA degradation during programmed cell death. These assays also provide the means by which to probe the relationship of DNA degradation to other aspects of programmed cell death, such as cell-corpse engulfment.

Assays for Gene Functions in Cell Death Using Transgenic Techniques

Transgenic animals are straightforward to generate in *C. elegans*. Transgene constructs are injected directly into the gonad of young adult hermaphrodites, usually with a marker plasmid such as pRF4, which creates a dominant Roller phenotype in transgenic animals.²¹ The germline in *C. elegans* gonad develops as a syncytium. As oocytes form, the injected DNA is packaged into the oocytes. The injected DNA does not usually integrate into chromosomes, but rather is maintained as an extrachromosomal array containing hundreds of copies of the gene injected.²¹ To generate animals with the transgene array integrated into one of the chromosomes, X-ray or UV light can be used to initiate breaks in chromosomes where the array can integrate.²² F2 progeny of irradiated animals are then screened for 100% inheritance of the transgene which

should segregate in crosses in a Mendelian fashion. Detailed protocols for generating transgenic nematodes can be found in other references.^{16, 21}

Transgenic techniques have been used to test whether expression of a gene (either endogenous or exogenous) under the control of specific promoters can interfere with the cell-death process in *C. elegans*. Genes whose activities are expected to cause cell death can be expressed in cells that are not essential for the viability of the animals. One commonly used promoter is the *mec-7* promoter, which drives gene expression in six mechanosensory neurons (AVM, ALML/R, PVM, and PLML/R) that mediate touch response in *C. elegans*.^{23, 24} The killing effect of a transgene is assessed by the death of the mechanosensory neurons in which the transgene is ectopically expressed. For example, expression of the cell-killing gene *ced-3* or *ced-4* in the touch cells using the *mec-7* promoter, causes the death of mechanosensory neurons.²⁵ The death of these neurons can be scored using Nomarski optics (e.g., deaths of the left and right ALM cells can be scored at the early L1 stage for the absence of ALM nuclei) and can be further confirmed by lineage analysis.²⁵ An alternative way to detect the death of the mechanosensory neurons is to co-express green fluorescent protein (GFP)²⁶ with the test gene in mechanosensory neurons using the *mec-7* promoter. The presence of GFP can then be used as an easy cell-survival marker (D. Ledwich and D. Xue, unpublished results). Other promoters that have been used for similar cell-death assays are the *unc-30* promoter,²⁵ which drives gene expression in VD and DD neurons at the ventral cord.²⁷

For genes that are implicated in inhibiting cell death, ectopic expression studies can be performed using broadly expressed promoters such as the heat-shock promoters and the *dpy-30* promoter,^{28, 29} as lack of cell death has no apparent deleterious effects on *C. elegans*.^{1, 6, 7} *C. elegans* heat-shock promoters are ideal for such a purpose since they are heat-inducible and are

expressed at high levels.²⁸ The combination of two different heat-shock promoters (*hsp16-2* and *hsp16-41*) can drive gene expression in most *C. elegans* somatic cells.²⁸ Following the heat-shock treatment, transgenic animals are examined for extra surviving cells as described above to assess the death inhibitory activity of the transgene. This method has been used in *C. elegans* to test several endogenous or exogenous cell-death inhibitors, including *C. elegans* cell-death inhibitor CED-9³⁰ and its human homolog Bcl-2,³¹ the long transcript of the *C. elegans ced-4* gene,²⁹ and the baculovirus cell-death inhibitor p35.^{32,33} These proteins have been shown to prevent cell death in *C. elegans* to different extents, with CED-9 having the strongest death-protective activity. Various *C. elegans* ectopic expression vectors including the expression vectors containing the *mec-7* promoter and the heat-shock promoters can be requested from the laboratory of Dr. Andy Fire at the Carnegie Institute of Washington (<ftp://ciw2.ciwemb.edu/pub/FireLabVectors>).

To carry out a cell survival assay on transgenic animals carrying heat-shock constructs, gravid adults are transferred to freshly seeded plates to lay eggs for one hour at 20°C. These plates are then moved to a 33°C incubator for the heat-shock treatment. After a 45-minute heat shock, the adult animals are returned to 20°C for recovery and to continue egg-laying for 75 minutes before they are removed from the plates. L3 or L4 larvae derived from the eggs remaining on the plates are scored for extra cells in the anterior pharynx.

***In vitro* Assays for Death Proteases**

One key member of the *C. elegans* cell-death pathway is the *ced-3* gene. Mutations in *ced-3* prevent most if not all programmed cell deaths in *C. elegans*. *ced-3* encodes a protein that is similar to members of mammalian caspase family, which are highly specific cysteine proteases

that cleave their substrates after aspartate.^{34,35} CED-3 and other caspases are first synthesized as inactive protease precursors and later activated through proteolysis to generate active proteases, which are heterodimers containing a large subunit around 20 kDa and a small subunit around 10 kDa.³⁵ One of the central questions in the cell-death field is how the activities of the CED-3/caspases are regulated and what are targets of these death proteases that mediate the cell-killing process. To address these questions, it is important to have an *in vitro* assay that can be used to quantitate the activities of the death proteases and to test potential substrates of these proteases.

Active death proteases, including CED-3, can be easily produced by overexpressing protease precursors in bacteria using robust bacterial protein expression vectors such as pET vectors (Novagen).¹⁹ To facilitate the purification of active proteases from bacteria, an epitope tag such as the His₆ tag or the FLAG tag (DYKDDDDK) is added to the amino terminus of the protease precursor and the active protease complex can be purified through affinity-chromatography using appropriate affinity columns.¹⁹

Two methods have been developed to measure the activities of purified proteases. One involves using peptide substrates that are covalently linked to a fluorogenic group such as AMC (7-amido-4-methylcoumarin). When fluorogenic substrates are incubated with the death proteases, cleavage of the substrates by the proteases releases the fluorogenic group, which emits fluorescence when excited at an appropriate wavelength. The resulting fluorescence can then be quantitated using a fluorescence spectrophotometer. The fluorescence intensity is proportional to the amount of peptide substrate cleaved and the activity of the caspase can be determined accordingly. For example, Ac-DEVD-AMC is a good fluorogenic substrate for the CED-3 protease (D. Xue, unpublished results). This assay is best used for studying the activities of death proteases (purified or unpurified) and the regulation of protease activities by potential

regulators. A typical fluorometric reaction for the CED-3 protease contains 10 ng of purified CED-3 protease and 5 μ M Ac-DEVD-AMC in 1 ml of CED-3 buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.5 mM sucrose, and 5% glycerol). The assay is conducted at 30°C for one hour. The reaction is then excited at 360 nm, and the intensity of emission is measured at 460 nm. The readout is compared with that of 5 μ M AMC control solution to determine the percentage of the fluorogenic peptide that is cleaved by CED-3 as a measurement of protease activity. Potential protease regulators (either purified or unpurified) can be added directly into the reaction to test whether they can alter the protease activity.

The second method involves incubating ³⁵S-Methionine labeled proteins with death proteases to measure the protease activities or to determine whether the labeled protein is a protease substrate.¹⁹ The procedures include synthesizing ³⁵S-Methionine labeled proteins using an *in vitro* transcription and translation coupled system (Promega), incubating labeled proteins with death proteases, resolving protease reactions on SDS-polyacrylamide gels, and detecting labeled protein products through autoradiography. This assay is best used for examining whether a protein is a substrate of death proteases. It can also be applied to measure activities of death proteases, albeit it is less quantitative than the fluorometric assay. A typical reaction of such assay contains 1.0 μ l (10 ng) of purified CED-3 protease, 1.0 μ l of ³⁵S-Methionine labeled protein, and 2.0 μ l of CED-3 buffer. The assay is conducted at 30°C for one hour.¹⁹ The protease reaction is terminated by adding 4.0 μ l of SDS gel loading buffer and then resolved on a 10% or 15% SDS-PAGE gel. The gel is dried and then subjected to autoradiography to determine the cleavage of the labeled substrate by the protease.

Concluding Remarks

The methods described above for studying programmed cell death in *C. elegans* have been instrumental for elucidating the functions of the cell-death genes and the mechanisms by which they affect programmed cell death. The opportunity to observe and follow the death of a single cell such as the death of NSM sister cells in live animals allows cell death to be studied *in vivo* at a level and resolution that no other systems can currently match. The assay for scoring extra cells in the anterior pharynx of *C. elegans* provides a quantitative measurement of defects in cell death. The cell-corpse assay helps to identify and characterize engulfment-defective mutants and the studies of such mutants will shed light into how dying cells interact with engulfing cells to coordinate the tightly regulated corpse-engulfment process. The *in vitro* protease assays enable genetic pathway of cell death to be analyzed at the biochemical and mechanistic levels.^{19, 33, 36} The soon-to-be-finished *C. elegans* genome sequencing project and the availability of reverse genetic approaches such as *C. elegans* deletion library screens for deletion mutations and the simple but powerful dsRNA interference technique open new avenues to study candidate genes that may be involved in *C. elegans* cell death (by their sequence homology to other cell-death proteins) but have escaped previous mutant hunts.

It should be noted, however, that some assays commonly used to study apoptosis in other organisms are not yet available or remain to be developed in *C. elegans*. For example, it is not yet possible to visualize the formation of DNA ladder (the hallmark of apoptosis) during *C. elegans* programmed cell death. Perhaps the percentage of cells that undergo programmed cell death at any given developmental stage is too low for the internucleosomal DNA fragmentation to be clearly detected. Other methods to detect chromosomal fragmentation in dying cells such as the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling) assay³⁷ are plausible and are currently under development (Y.-C. Wu, G. M. Stanfield, and H. R.

Horvitz, personal communication). Assays that assess the integrity of plasma membrane, such as trypan blue exclusion assay,³⁸ or assays that assess the polarity of the lipid bilayer of plasma membrane, such as Annexin V labeling assay,³⁹ remain to be tested in *C. elegans*. Development of these cell-death assays and other new assays can further facilitate the study of the mechanisms of programmed cell death in *C. elegans* and will certainly contribute to the understanding of apoptosis in other organisms.

Figure Legends

Figure 1. Assay for Scoring Extra Nuclei in the Anterior Pharynx of *C. elegans*. (A) Schematic representation of the nuclei located within the anterior pharynx examined in the assay. Nuclei (open) of living cells and nuclei (shaded) of sixteen cells that normally undergo programmed cell death in wild-type animals are shown. The two NSM neurons and their sister cells are indicated. (B) Nomarski photomicrograph of the anterior pharynx of a *ced-3(n717)* mutant animal (left side). Several nuclei of the cells that normally live in wild-type animals are indicated by arrowheads. One extra nucleus is indicated by an arrow. (C) Nomarski photomicrograph of the anterior pharynx of a *ced-3(n717)* mutant animal (right side). An extra nucleus from the NSM sister cell located posterior and dorsal to the nucleus of the NSM neuron (arrowhead) is indicated by an arrow. The positions of the nuclei indicated in (B) and (C) are also pointed out in (A) using similar labels. The scale bar represents 10 μm .

Figure 2. Cell Corpses in Wild-Type Animals Show a Characteristic Morphology. (A) Nomarski micrograph of an embryo at two-fold stage. One raised-button-like somatic cell corpse is

indicated by an arrow. (B) Nomarski micrograph of a posterior gonadal arm. One germline cell corpse is indicated by an arrow. The scale bar represents 10 μ m.

Figure 3. Engulfment-Defective Mutants Exhibit Persistent Cell Corpses. (A) Nomarski micrograph of the head of a wild-type L1 larvae that lacks cell corpses. (B) Nomarski micrograph of the head of a *ced-1(e1735)* mutant at L1 larval stage with many cell corpses (indicated by arrows). The scale bar represents 10 μ m.

Figure 4. Schematic demonstration of making an agar pad.

Acknowledgment:

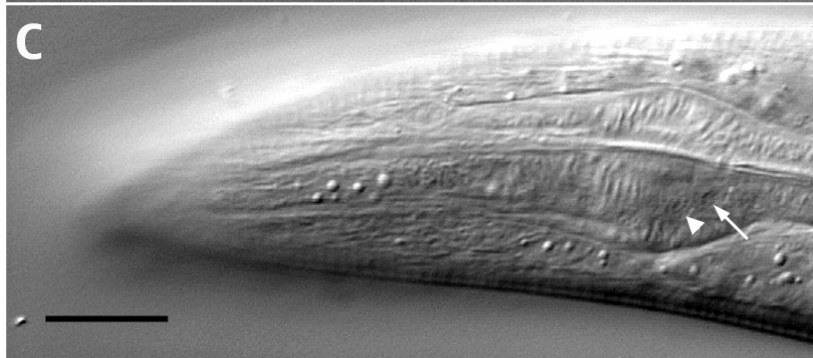
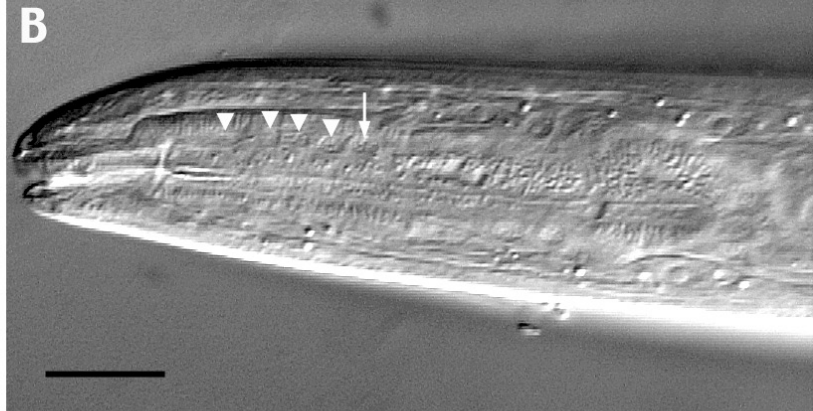
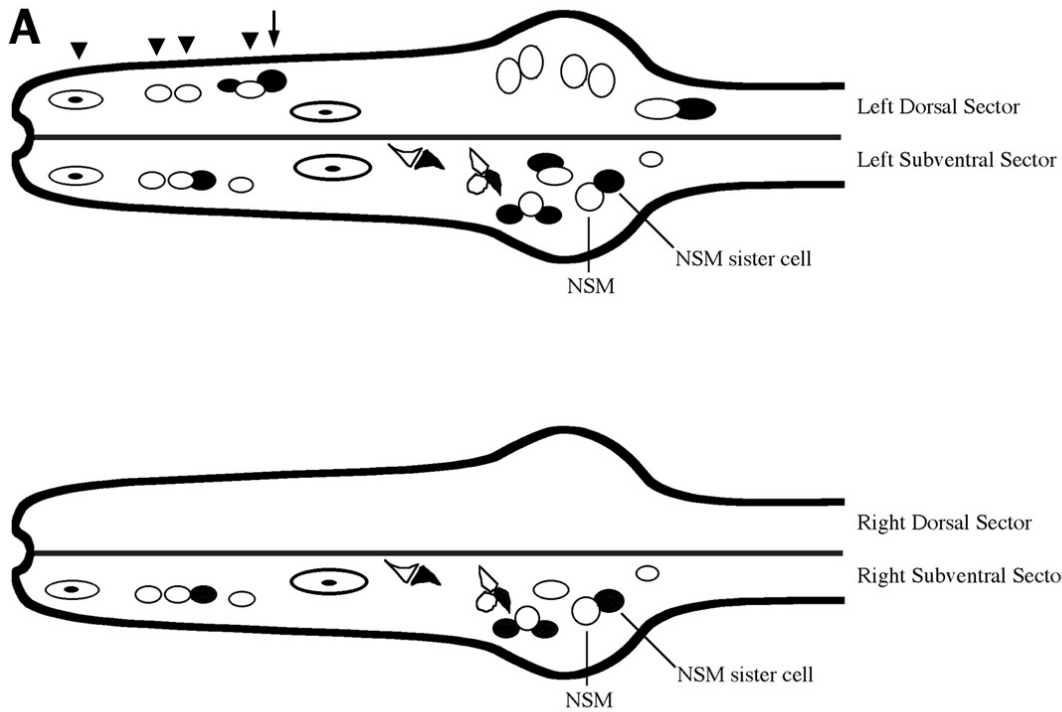
We thank J. Lee for comments on this manuscript. D. Xue is a recipient of a Burroughs Wellcome Fund Career Award in the Biomedical Sciences.

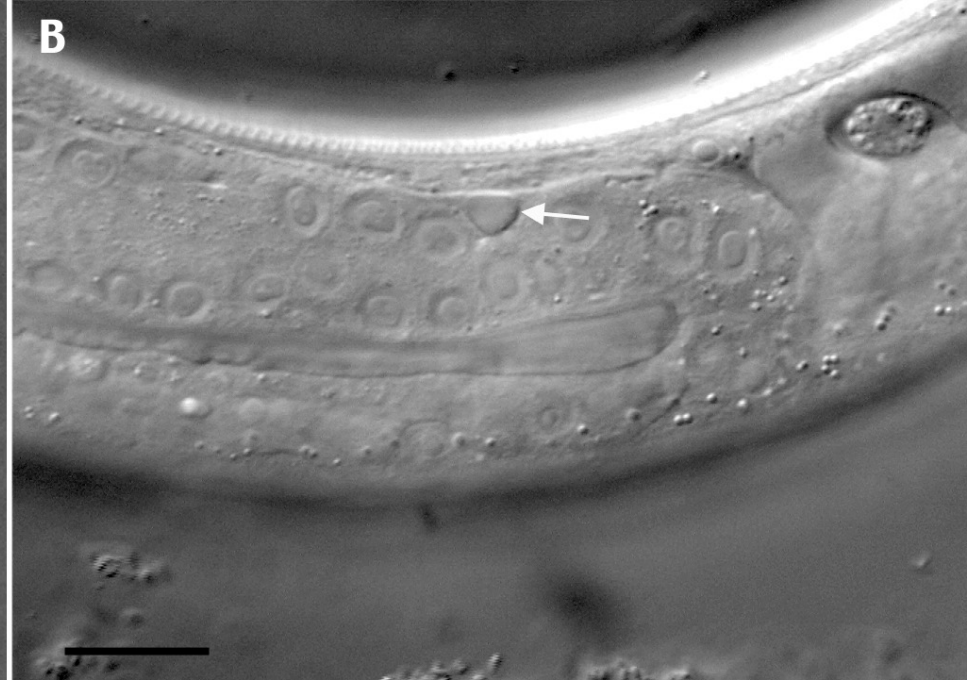
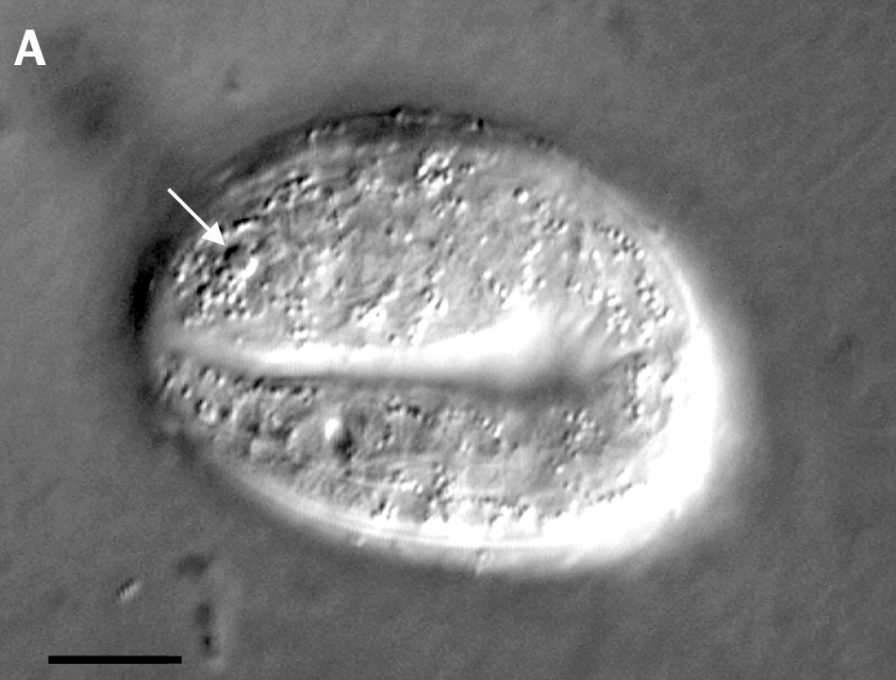
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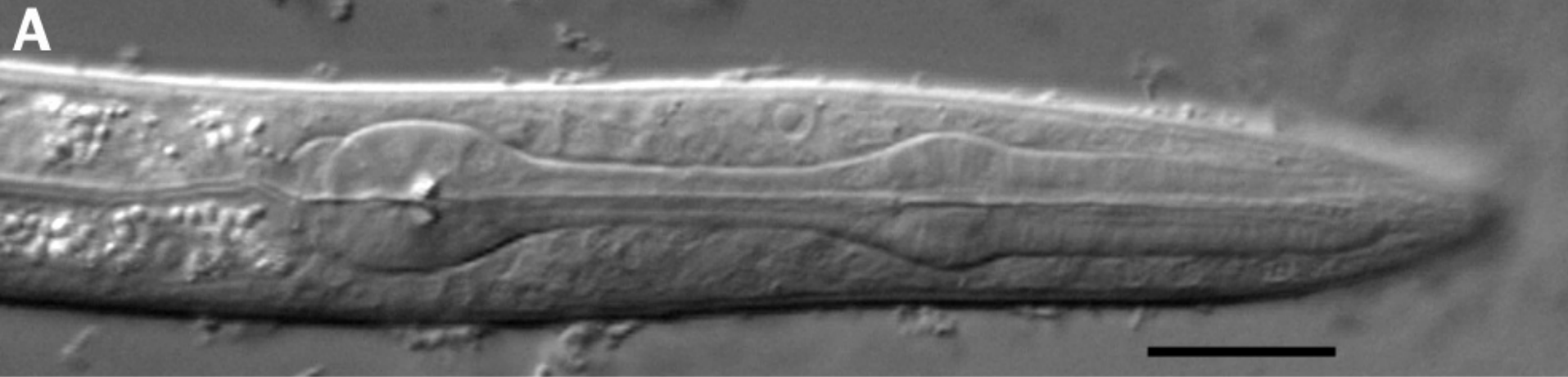
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